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Sequence Requirements for Incorporation of Human Immunodeficiency Virus Gag-β-Galactosidase Fusion Proteins Into Virus-Like Particles

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The incorporation of human immunodeficiency virus type 1 (HIV-1) Gag-β-galactosidase (Gag-β-gal; GBG) fusion proteins into HIV virus-like particles in the presence of HIV Gag proteins was studied. HIV Gag-β-gal fusion constructs were cotransfected individually into COS7 cells with or without an HIV Gag protein expression plasmid. Release of HIV GBG fusion proteins from the cells were measured by assay of the medium versus intracellular β-gal activities. Analysis indicates that fusion proteins (constructs HIVGBG, GBG 1919 and 1877) retaining the C-terminal portion of the CA and the adjacent NC domains were efficiently assembled into virus-like particles. Fusion proteins with deleted sequences covering the N-terminal portions of the *gag* sequences (GBG 831, 1147, 1419, 1447, 1511, 1552, 1600, 1630, 1684, 1715, and 1752) were impaired in entry into virus-like particles. The presence of CA major homology region (MHR) in the fusion proteins had no significant effects on inducing fusion protein incorporation when the C-terminal CA sequences in the fusion proteins were truncated (GBG 1841 and 1801). Subcellular fractionation studies indicated that most fusion proteins including the nonmyristylated one were enriched in the crude membrane fraction. Exceptions to this rule were fusion proteins with intact MHR but truncated C-terminal CA sequences, which possessed low levels of membrane association. However, assembly of fusion proteins into HIV Gag particles did not correlate with their subcellular fractionation or immunofluorescence localization patterns. Overall, the studies suggest that the very C-terminal CA and adjacent NC sequences are the primary determinants for incorporation of HIV Gag-β-gal fusion proteins into virus particles. ***J. Med. Virol. 59:180–188, 1999.***

KEY WORDS: human immunodeficiency virus; Gag; Gag-β-gal fusion proteins

INTRODUCTION

The human immunodeficiency virus (HIV) structural protein Gag contains sufficient information for particle formation [Gheysen et al., 1989; Karacostas et al., 1989; Wagner et al., 1994]. Assembly of HIV Gag particles occurs at the plasma membrane where Gag molecules self-assemble into virus particles and bud out from the cell membrane [Stephens and Compans, 1988; Cann and Karn, 1989; Gelderblom, 1991]. The Gag protein is initially translated as a precursor $Pr55^{gag}$. During translation, the N-terminal methionine is removed and a myristic acid is attached to the second glycine residue [Towler et al., 1987; Wilcox et al., 1987]. Myristylation of $Pr55^{gag}$ is required for particle assembly and release [Gottlinger et al., 1989; Bryant and Ratner, 1990; Pal et al., 1990]. During or after virus budding [Kaplan et al., 1994], the $Pr55^{gag}$ is cleaved by the viral protease into p17 (matrix domain; MA), p24 (capsid domain; CA), p2, p7 (nucleocapsid domain; NC), p1, and the C-terminal p6 peptide [Leis et al., 1988; Mervis et al., 1988; Overton et al., 1989; Henderson et al., 1992]. The myristylated MA domain is intimately associated with cell membrane [Zhou et al., 1994] and has been shown to mediate the envelope protein incorporation [Yu et al., 1992; Dorfman et al., 1994a]. The CA domain is the major capsid protein. A highly conserved region among retrovirus Gag domains referred to as major homology region (MHR) is located in the C-terminal CA and is important for particle assembly [Hong and Boulanger, 1993; von Poblotzki et al., 1993; Mammano et al., 1994; Dorfman et al., 1994b; Reicin et

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al., 1995; Gamble et al., 1997]. The NC domain contains two Cys-His motifs that are required for viral genomic RNA packaging [Aldovini and Young, 1990]. The p6 domain is postulated to be involved in the process of virus budding [Gheysen et al., 1989; Gottlinger et al., 1989] and has been shown to be required for the incorporation of the accessory protein Vpr into particles [Lu et al., 1993; Paxton et al., 1993].

Enzymes required for virus replication are encoded by the *pol*, which is translated as a precursor $Pr160^{gag\text{-}pol}$ by −1 ribosomal frameshifting. The ribosomal frameshifting event occurs at a frequency of 5%–10% during translation of Gag [Jacks et al., 1988; Wilson et al., 1988]. Within the Gag-Pol precursor, the p6 is truncated and replaced by a peptide referred to as P6* [Partin et al., 1990]. Proteolytic cleavage of Pol yields protease (PR), reverse transcriptase (RT), Rnase H, and integrase (IN) [Ratner et al., 1985]. $Pr160^{gag\text{-}pol}$ appears to be incorporated into virions via interaction with the assembling $Pr55^{gag}$ particles [Wills and Craven, 1991]. Although the CA domain within the Gag-Pol is thought to play a pivotal role in the process of Gag-Pol incorporation into virions [Huang and Martin, 1997], regions responsible for Gag-Pol assembly into particles have not been defined precisely. Recent studies have found that the myristylation signal essential for $Pr55^{gag}$ assembly is not absolutely required for $Pr160^{gag\text{-}pol}$ assembly into virus particles [Park and Morrow, 1992; Smith et al., 1993]. In addition, one study with $Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$ expressed individually from separate plasmid suggested that substitution mutations in MHR within the Gag-Pol context have no major effects on $Pr160^{gag\text{-}pol}$ incorporation into wild-type $Pr55^{gag}$ particles [Mammano et al., 1994]. However, the other study with the similar approach showed that deletion mutations in MHR would exclude the incorporation of mutant $Pr160^{gag\text{-}pol}$ into wild-type $Pr55^{gag}$ particles [Srinivasakumar et al., 1995].

We adapted Gag-β-galactosidase (GBG) fusion protein expression system established previously [Wang et al., 1994] to study the incorporation of GBG proteins into HIV virus particles. In the system, a plasmid construct designated HIVGBG was generated by replacement of the HIV *pol* gene with the β-gal gene. On coexpression with HIVgpt in COS7 cells, GBG fusion proteins could be incorporated into virions, which was measured by assay of media versus intracellular β-gal activities. This system provides a convenient assay for mapping the domains involved potentially in the incorporation of $Pr160^{gag\text{-}pol}$ into virus particles. To define in more details the regions required for assembly of HIV Gag-β-gal fusion protein into particles, a series of Gag-β-gal fusion constructs was made by fusing the β-gal gene to various regions of the HIV *gag* gene. Each GBG fusion construct was expressed in the presence or absence of wt HIV Gag protein, and incorporation of the GBG fusion protein into virions was assessed by the level of released β-gal activity. The results showed that fusion proteins containing the HIV MA and CA were efficiently assembled into virions, while fusion proteins with deletions covering the very C-terminus of CA domain are incorporated into virus particles at low efficiency. Subcellular fractionation studies suggested that the ability of GBG fusion proteins to assemble into virions was relatively independent of fractionation patterns, and did not correlate with immunofluorescene staining patterns.

MATERIALS AMD METHODS

Plasmid Construction

Plasmids HIVgpt [Page et al., 1990] and HIV Gag-β-gal fusion constructs HIVGBG, GBG Myr^-, GBG 831, and GBG 1147 were as described previously [Wang et al., 1994]. Briefly, the HIVGBG was constructed by replacement of the BglII (HXB2 nt 2096) to SalI (HXB2 nt. 5786) fragment in HIVgpt with a BamHI-SalI fragment containing the β-galactosidase coding region from a modified BAG vector [Jones et al., 1990]. The sequence at the junction area is AG ATC TCG GGG GAT CCC GTC. The underlined nucleotide is HIV nt. 2096 and the fusion site is a BamHI linker adjacent to the eighth codon (GTC) of the β-gal coding region. Introducing the myristylation-minus HIV *gag* mutant [Wang and Barklis, 1993] into the HIVGBG construct generated nonmyristylated GBG (GBG Myr^-). The other fusion protein constructs 831, 1147, 1419, 1447, 1511, 1552, 1600, 1630, 1684, 1715, 1752, 1801, 1841, 1877, and 1919 were made by using a BamHI linker and were fused with the β-gal gene at the designated HIV-1 nt position (Fig. 1). BamHI linkers were created either by linker insertion or by overlap-extension PCR [Ho et al., 1989; Horton et al., 1989]. Methods for cloning and DNA sequencing followed the protocol as described by Sambrook et al. [1989]. The sequences at the fusion junctions are as follows, where the underlined nt indicates the HIV nt position designated in the construct name, and the final GTC is the eighth codon of β-gal: 831, CGA TTA GAT CCC GTC; 1147, CG GAT CCC GTC; 1419, GGC GGC CGC ACT AGT GAT TCG GAT CCC GTC; 1447, CT CCC GGC CGC CAT GGC CGC GGG ATT CGG GAT CCC GTC; 1511, T GAT TCG GAT CCC GTC; 1552, CG GAT CCC GTC; 1600, AG GAT TCC GTC; 1630, GG ATC TC G GGG GAT CCC GTC; 1684, GG ATC TCG GGG GAT CCC GTC; 1715, T CGG GAT CCC GTC; 1752, TGG ATC TCG GGG GAT CCC GTC; 1801, GG ATC TCG GGG GAT CCC GTC; 1841, G GAT CCC GTC; 1877, G GAT CCC GTC; 1919, G GAT CCC GTC.

Cell Culture and Transfections

COS7 cells were maintained in DMEM supplemented with 10% fetal calf serum. Confluent COS7 cells were trypsinized and split 1:10 onto 10-cm dish plates 24 hours before transfections. Fifteen micrograms of plasmid DNA of HIV Gag-β-gal constructs

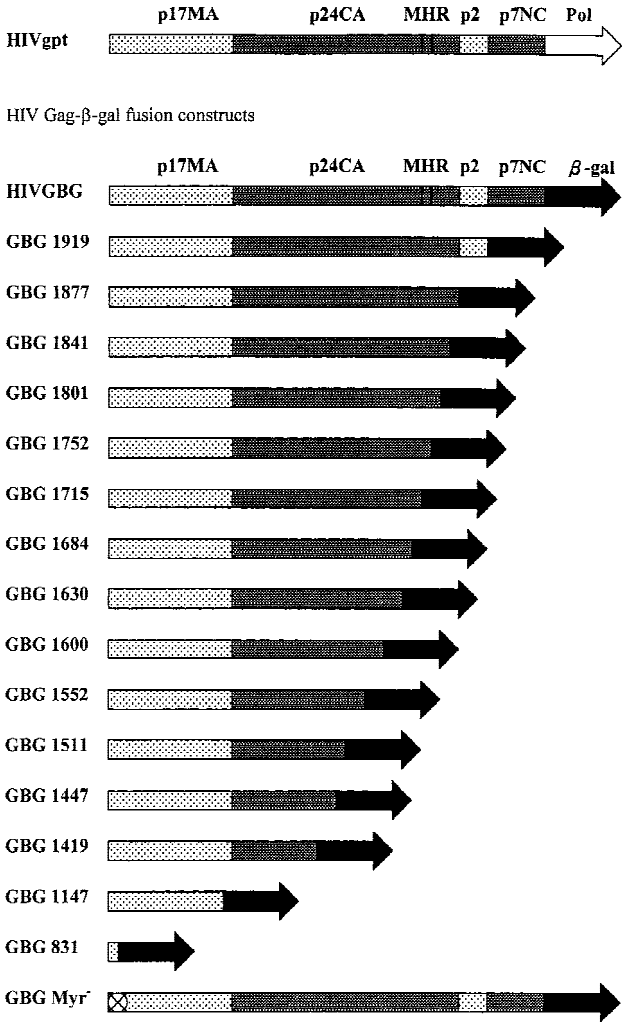

Fig. 1. Recombinant constructs. HIVgpt construct [Page et al., 1990]: the wt *gag* domains p17 (matrix; MA), p24 (capsid, CA), p2, p7 (nucleocapsid, NC), and the major homology region (MHR) spanning nt 1641-1694 are illustrated. HIVGBG constructs: as described in the Materials and Methods, the Gag-β-galactosidase fusion constructs were made by replacing regions from the indicated *gag* nt locations to the HIVgpt SalI site (HXB2 nt 5786) with β-gal. GBG 1919 is fused in the nucleocapsid (NC) region; GBG 1877 through 1419 are fused in the capsid (CA)-coding regions; GBG 1147 and 831 are fused in the matrix (MA) region. For GBG Myr$^-$, the fusion site was as the HIVGBG but the second amino acid glycine of Gag was changed to alanine, blocks the Gag myristylation [Bryant and Ratner, 1990; Wang and Barklis, 1993].

were transfected into COS7 cells by calcium phosphate precipitation. For cotransfection with HIVgpt, 10 μg of each plasmid DNA were used. At 48–72 hours after transfection, cells and culture media were collected for protein analysis or for β-gal enzyme assay.

Protein and β-gal Activity Assays

Culture media from transfected COS7 cells were filtered through a 0.45 μm-pore-size filter and subjected to ultracentrifugation at 4°C for 40 min at 40,000 rpm. Pellets were suspended in 200 μl of 1X phosphate-buffered saline (PBS). Cells were washed twice with ice-cold 1× PBS and collected in 1 ml of 1× PBS, pelleted and resuspended in 1X PBS. β-gal assay of medium and cell samples were conducted according to Norton and Coffin [Norton and Coffin, 1985]. Total cellular proteins were quantitated by the microassay method of Bradford [1976].

Western Immunoblot and Immunofluorescence

For immunoblot experiment, cell pellets were lysed in IPB (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton-X 100, 0.02% sodium azide). Cell lysates then were centrifuged at 4°C for 15 min at 14,000 rpm to remove cell debris. Supernatants were mixed with equal volumes of 2× sample buffer (12.5 mM Tris-HCl [pH 6.8], 2% SDS, 20% glycerol, 0.25% bromophenol blue) plus 5% β-mercaptoethanol, boiled for 5 minutes, subjected to SDS-PAGE, and electroblotted onto nitrocellulose membranes. Procedures for immunodetection of membrane-bound fusion proteins were followed as previously described [Chen et al., 1997; Wang et al., 1998]. A mouse anti-β-galactosidase monoclonal antibody (Boehringer Mannheim) at 1:1000 dilution was used as primary antibody. For detection of HIV Gag antigens, the primary antibody was an anti-p24^{gag} monoclonal antibody (mouse hybridoma clone 183-H12-5C) and used at a 1:5,000 dilution. The secondary antibody was a sheep anti-mouse alkaline phosphatase-conjugated IgG antibody (Vector Laboratories). The protocol for indirect immunofluorescence experiment was as described previously [Wang and Barklis, 1993]. The primary antibody was an anti-β-gal monoclonal antibody at 1:1000 dilution and the secondary antibody was a rabbit anti-mouse rhodamine-conjugated IgG antibody at 1:100 dilution (Cappel).

Sucrose Density Gradient Fractionation

Culture supernatants of transfected COS7 cells were collected, filtered, and centrifuged through 2-ml 20% sucrose cushions at 4°C for 40 minutes at 274,000 *g*. Pellets than were suspended in TSE (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA) and overlaid on top of premade 20% to 60% sucrose gradients consisting of 1-ml layers of 20, 30, 40, 50, and 60 sucrose in TSE which had been allowed to mix by sitting for 2 hours. Gradients were centrifuged at 274,000*g* for 16– 18 hours at 4°C, and 500-μl fractions were collected from top to bottom. Each fraction was measured for density and analyzed for β-gal activities and for Gag proteins by Western immunoblotting.

Stability of Intracellular Gag-β-gal Fusion Proteins

COS7 cells transfected with GBG constructs were trypsinized 24 hours after transfection, split equally onto 6-cm dish plates and grown for 18 hours in DMEM plus 10% fetal calf serum. Culture medium then was removed and the cells were refed with medium containing 100 μg/ml cycloheximide, after which cells were collected at various time points for β-gal assays.

Subcellular Fractionation

At 48–72 hours after transfection, COS7 cells were collected, pelleted, and resuspended in 1.5 ml swelling buffer (2 mM $NaHCO_3$, 1 mM $MgCl_2$, and 0.1 mM PMSF), and set on ice for 5 min followed by douncing 200 times with a Wheaton dounce type A pestle. Cell lysates were cleared of unbroken cells, nuclei, and debris by centrifugation at 2,500 rpm for 5 minutes. Supernatants then were fractionated at 174,000*g* at 4°C for 15 minutes, after which supernatants (S2) and pellets (P2) representing crude cytosol and membrane fraction respectively were collected for total protein and β-gal assays.

RESULTS

Construction of HIV Gag-β-gal Fusion Constructs

An HIV Gag-β-gal (GBG) fusion protein system was used to investigate the Gag domain required for HIV Gag-β-gal fusion protein assembly into particles. To do so, a series of HIV Gag-β-gal fusion constructs were generated as described in the Materials and Methods section. For the analysis, seventeen fusion constructs were obtained, including four constructed previously, fusion plasmids HIVGBG, GBG 1147, GBG 831, and GBG Myr^- [Wang et al., 1994]. These fusion constructs retain the same N-terminal coding sequence of the *gag* but differ in the extent of *gag* coding region fused to the β-gal gene. As illustrated in Figure 1, GBG 1919 was generated by β-gal fusion at the end of p2; GBG 1877, 1841, 1801, 1752, 1715, 1684, 1630, 1600, 1552, 1511, 1447, and 1419 were fused in the CA region; GBG 1147 and 831 were fused in the MA region; GBG Myr^- was identical to HIVGBG except that it contained a myristylation-deficient mutation in the *gag*. Note that fusion constructs GBG 1684 through 831 each carries a truncated or deleted MHR.

Expression and Incorporation of HIV Gag-β-gal Fusion Proteins Into Virus Particles

Each fusion plasmid construct was transfected transiently into COS7 cells and expression of the HIV Gag-β-gal fusion protein was detected by Western immunoblotting using an anti-β-gal monoclonal antibody. As shown in Figure 2, the HIVGBG Gag-β-gal fusion protein was detected of about 160 kDa in size (lane 1), while the GBG 831 fusion protein was detected as a band of 110 Kda (lane 2), corresponding to β-gal fusion at HIV nt 2096 and 831, respectively. Fusion proteins of GBG 1147 (lane 3), GBG 1419 (lane 4), GBG 1552 (lane 5), GBG 1630 (lane 6), GBG 1715 (lane 7), and GBG 1841 (lane 8) were observed readily as bands of predicted molecular weights. Expression of the other GBG fusion constructs 1511, 1600, 1684, 1752, 1801, 1877, 1919, and Myr^- was also confirmed by Western immunoblotting (data not shown).

To assess the incorporation of Gag-β-gal fusion proteins into HIV particles, the protocol shown in Figure 3 was used. As illustrated, the β-gal fusion constructs

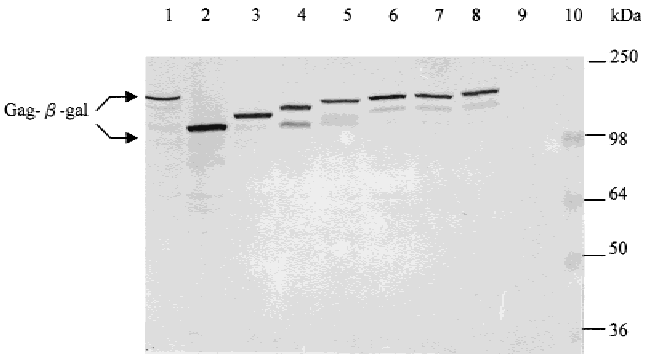

Fig. 2. Transient expression of HIV Gag-β-gal fusion proteins in COS7 cells. At two to three days after transfection, COS7 cells were collected and prepared for Western immunoblotting analysis as described in the Materials and Methods section. Cell samples (corresponding to 5% of the total cell sample) were subjected to SDS-7.5% polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Gag-β-gal fusion proteins were detected by a primary anti-β-gal monoclonal and a secondary alkaline phosphatase-conjugated sheep anti-mouse antibody, followed by detection of alkaline phosphatase activity. Molecular size markers (**lane 10**) are indicated on the right. **Lanes: 1**, HIVGBG; **2**, GBG 831; **3**, GBG 1147; **4**, GBG 1419; **5**, GBG 1552; **6**, GBG 1630; **7**, GBG 1715; **8**, GBG 1841; **9**, Mock; 10, Standard.

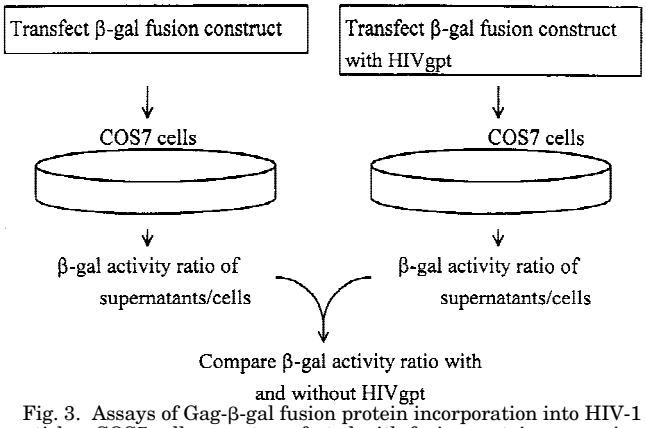

Fig. 3. Assays of Gag-β-gal fusion protein incorporation into HIV-1 particles. COS7 cells were transfected with fusion protein expression constructs with or without HIVgpt. Three days after transfection, media and cells were collected and assayed for β-gal activity as described in the Materials and Methods section. For each transfection, ratios of β-gal activity levels in the media versus in the cells indicated the level of released HIV Gag-β-gal fusion proteins. β-gal release ratios in the presence versus absence of Gag proteins expressed from HIVgpt were compared in parallel transfections. Higher release levels in the presence of HIVgpt helper construct are indicative of Gag-β-gal fusion protein incorporation into virus particles.

were transfected into COS7 cells with or without the HIVgpt and the amount of released Gag-β-gal fusion proteins were monitored by the ratios of the extracellular versus intracellular β-gal enzyme activities. Without coexpression of HIVgpt, the ratios of media to cellular β-gal activities produced by HIV Gag-β-gal fusion constructs were at the range between 0.013–0.039, indicating very low levels of β-gal activity release (Table I). In contrast, there was an over 10-fold increase in β-gal release when HIVGBG was co-transfected with the HIVgpt. To confirm that the β-gal activity is particle-associated, sucrose density gradient fractionation experiments were undertaken as de-

TABLE I. Requirements for Fusion Proteins Incorporation into HIV Virus Particles

Gag-β-gal constructs	*N*	(Virus β-gal/cell β-gal) −HIVgpt	(Virus β-gal/cell β-gal) +HIVgpt	Ratio
HIVGBG	14	0.017 ± 0.017	0.195 ± 0.117	18.101 ± 15.378
GBG 1919	5	0.031 ± 0.018	0.156 ± 0.077	8.503 ± 7.949
GBG 1877	5	0.039 ± 0.039	0.129 ± 0.025	7.994 ± 7.824
GBG 1841	5	0.020 ± 0.010	0.080 ± 0.020	4.286 ± 1.780
GBG 1801	3	0.017 ± 0.014	0.041 ± 0.006	3.558 ± 2.387
GBG 1752	3	0.020 ± 0.016	0.025 ± 0.004	1.709 ± 0.908
GBG 1715	6	0.019 ± 0.008	0.042 ± 0.015	2.467 ± 1.370
GBG 1684	3	0.018 ± 0.010	0.025 ± 0.008	1.590 ± 0.678
GBG 1630	4	0.030 ± 0.013	0.035 ± 0.013	1.313 ± 0.637
GBG 1600	6	0.015 ± 0.010	0.030 ± 0.017	2.608 ± 2.105
GBG 1552	7	0.027 ± 0.005	0.063 ± 0.018	2.413 ± 0.732
GBG 1511	4	0.021 ± 0.014	0.040 ± 0.007	2.572 ± 1.449
GBG 1447	4	0.020 ± 0.014	0.040 ± 0.059	1.477 ± 1.198
GBG 1419	3	0.013 ± 0.003	0.029 ± 0.013	2.098 ± 0.693
GBG 1147	3	0.023 ± 0.010	0.025 ± 0.009	1.135 ± 0.273
GBG 831	2	0.016 ± 0.007	0.015 ± 0.011	0.866 ± 0.324
GBG Myr	2	0.024 ± 0.023	0.140 ± 0.042	3.760 ± 0.140

COS7 cells were transfected with Gag-β-gal expression constructs (Fig. 1) with or without the indicated HIVgpt helper construct, and the procedure for assay of Gag-β-gal fusion protein incorporation into virus particles was as illustrated in Figure 3 and described under Materials and Methods. Media and cell samples were aliquoted for β-gal enzyme activity assays. Results were presented as the ratios of total normalized β-gal enzyme activities released to the media versus inside cells with standard deviations. *N* indicates the number of times the experiment was performed. Data shown in the ratio column were obtained by comparing the ratios of β-gal activities in the presence versus absence of HIVgp, and were averaged from individual tabulated experimental ratios.

scribed in the Materials and Methods nsection. The results indicate that both the peak Gag protein and β-gal activity levels banded at fractions with densities of 1.16–1.18 g/ml (data not shown), which are consistent with the previous report and support the conclusion that the Gag-β-gal fusion proteins are incorporated into HIV Gag particles [Wang et al., 1994].

To test the ability of Gag-β-gal fusion protein to be incorporated into Gag particles, each Gag-β-gal fusion construct was cotransfected with or without HIVgpt and assembly of Gag-β-gal fusion proteins into particles was assayed as described above (Fig. 3). As shown in Table 1, deletion of the NC domain (GBG 1919) or deletion of the NC plus p2 domains (GBG 1877) effected a decrease in the β-gal release as compared with the HIVGBG; however, the GBG 1919 and 1877 still showed an 8-fold increase in β-gal release ratios in the presence of HIVgpt. GBG 1841 and 1801 showed a 3- to 4-fold increase in β-gal release ratios when coexpressed with HIVgpt, suggesting that GBG 1841 and 1801 were still able to enter virions. By contrast, fusion proteins produced by constructs GBG 1752 through 1147, lacking the C-terminal CA regions and the downstream p2 and NC domains, all exhibited a low virion entry efficiency. These results indicate that regions of the very C-terminal portions of CA and the adjacent NC domain are more important than the other areas to Gag-β-gal fusion protein incorporation into HIV particles. While fusion proteins of GBG 831 carrying a few amino acids of the N-terminal matrix protein showed no enhancement of β-gal release in the presence of HIVgpt helper, myristylation-minus version of HIVGBG, GBG Myr$^-$, demonstrated a 3.7-fold increase in β-gal release ratios. These data agreed with the previous report that nonmyristylated HIV Gag-β-gal fusion protein can be incorporated into virus particles [Wang et al., 1994].

Since the β-gal release ratios were derived from the media versus intracellular β-gal activity levels, different half-lives of intracellular and extracellular β-gal activities would affect the β-gal release ratios. However, it was found that the extracellular virus-associated β-gal appeared to be stable during incubation at 37°C over 24 hours (data not shown). To measure the half-life of intracellular β-gal activity, Gag-β-gal fusion constructs were transfected into COS7 cells and treated with cycloheximide to stop protein synthesis, after which intracellular β-gal activities were assayed over a time course. It was found that most fusion proteins have β-gal activity half-lives around 11–21 hours (data not shown). However, it appears unlikely that these intracellular β-gal activity half-life differences could adjust their β-gal release ratio to normal levels.

Intracellular Localization of Gag-β-gal Fusion Proteins

Because virus particle assembly occurs at the plasma membranes, failure of the GBG fusion protein to be associated with membrane or to be transported to the plasma membranes may account in part for the fusion proteins inability to be assembled into virus particles. We performed subcellular fractionation experiments to determine whether the intracellular Gag-β-gal fusion

TABLE II. Membrane Association of Fusion Proteins

β-gal construct	Total β-gal ratio (P2 β-gal/S2 β-gal)	Membrane association (P2 β-gal/P2 protein)/ (S2 β-gal/S2 protein)
HIVGBG	2.07	6.72
1919	1.96 ± 0.30	8.86 ± 5.13
1877	2.98 ± 0.08	10.69 ± 2.21
1841	4.37 ± 2.59	15.86 ± 10.81
1752	0.12 ± 0.11	0.41 ± 0.42
1715	0.18 ± 0.17	0.68 ± 0.74
1684	1.01	3.63
1600	0.83	3.64
1511	0.92	3.86
1447	1.13	4.39
1419	1.34	4.34
1147	0.98 ± 0.39	4.80 ± 0.99
831	1.90	4.80
Myr	0.58 ± 0.21	1.72 ± 0.64

At 48–72 hours after transfection with the designated constructs, COS7 cells were collected and fractionated into a crude membrane fraction, P2, and a cytosolic fraction, S2, as described in the Materials and Methods Section Fractions were assayed for total β-gal activities (units) and proteins levels (mg). The ratios of P2 versus S2 β-gal activities are indicated in the center column while the right-hand column shows the ratios of P2 versus S2-specific β-gal activities, with standard deviations when possible. Myr and 1147 values derive from three independent trials; 1919, 1877, 1841, 1752, and 1715 are from two experiments each; all other values derive from one trial each.

proteins were associated with intracytoplasmic membranes. To do so, transfected COS7 cells were fractionated to isolate the crude cellular membrane (P2) and cytosol (S2) fractions, which were subjected to β-gal activity and protein assay as described in the Materials and Methods section. Ratios of P2 versus S2 β-gal activity and specific β-gal activities were shown in Table II. Except for GBG 1752 and 1715, all the fusion proteins were associated with membrane to a certain extent with P2/S2-specific activity ratios from 1.72 to 15.86. However, we found no correlation between P2/S2 ratios and the fusion protein's ability to be incorporated into particles. For instances, both HIVGBG and GBG 831 had the P2/S2 ratios around 5-6, but HIVGBG can and GBG 831 cannot assemble into HIV particles.

To obtain more information about intracellular distribution of Gag-β-gal fusion proteins, indirect immunofluorescence experiments were conducted using an anti-β-gal monoclonal antibody as described in the Materials and Methods section. As shown in Figure 4, HIV GBG (Fig. 4A) and GBG Myr$^-$ (Fig. 4L) fusion proteins showed a similar pattern with a heterogeneous staining pattern and a clear perinuclear ring. However, the immunofluorescenc staining pattern did not correlate with the levels of fusion protein incorporation into virus particle as HIVGBG were efficiently incorporated into virions while GBG Myr$^-$ was not. The short fusion GBG 831 displayed an intense immunofluorescence staining in the perinuclear area (GBG 831; Fig. 4K), indicating an impairment in fusion protein transportation. This result correlates with the fusion protein's inability to be incorporated into virus particles. Interestingly, fusion proteins with intact MA but deleted NC domains (GBG 1919, 1877, 1801, 1752, 1715, 1600, 1511, 1447, and 1147) all appeared similar, with immunofluorescene staining throughout the cells and absence of clear perinuclear rings (Fig. 4B, 4C, 4D, 4E, 4F, 4G, 4H, 4I, and 4J, respectively). The other fusion constructs GBG 1841, 1684, 1630, 1552, 1419 also had a similar staining pattern (data not shown). Approximately 60–90% of cells transfected with fusion constructs that contained intact MA but deleted NC domains displayed this putative surface staining pattern while the other transfectants had a staining pattern similar to that of HIVGBG transfectants. This result suggests that the NC domain may be involved in compartmentalization of HIV Gag-β-gal fusion proteins in transfected cells.

DISCUSSION

Although the GBG fusion protein does not mimic the Pr160$^{gag\text{-}pol}$ completely in the process of incorporation into virus particles, the two appear to be drawn into assembling virions by virtue of interacting with the Pr55^{gag}. Previous studies have shown that the CA domain is important to both HIV Gag-β-gal [Wang et al., 1994] and Gag-Pol fusion protein [Huang and Martin, 1997] incorporation into virus particles. In addition, the Pr160$^{gag\text{-}pol}$ is similar to Gag-β-gal fusion proteins in that nonmyristylated versions of the two fusion proteins can assemble into virus particles [Park and Morrow, 1992; Smith et al., 1993]. Results from analysis of the incorporation of HIV Gag-β-gal fusion proteins into Gag particles may help define which regions of the Gag are important to the incorporation of Pr160$^{gag\text{-}pol}$ into virus particles.

Unlike murine leukemia virus (MLV) Gag-β-gal fusion proteins which are able to be released from the cells to the media in the absence of Gag proteins [Hansen et al., 1990; Jones et al., 1990], the HIV Gag-β-gal fusion proteins can not be released without the presence of HIV Gag proteins. On cotransfection with an HIV Gag protein expression plasmid HIVgpt, most of our fusion construct proteins were able to be incorporated into virions to some extent. Fusion proteins with intact MHR but deleted C-terminal CA (GBG 1841 and 1801) sequences were incorporated into virus particles at a relatively reduced level as compared with that of fusion proteins that retained intact CA domains (GBG1877 and 1919). Surprisingly, fusion proteins GBG 1752 and 1715 possessing an intact MHR did not appear to enter virus particle more efficiently than fusion proteins 1600, 1552 and 1511, which lacked the MHR sequences. These results imply that the MHR in the Gag-β-gal context contributes little to fusion protein incorporation in the absence of an intact CA domain and that both the MHR and the adjacent C-terminal CA regions are required for Gag-β-gal fusion protein incorporation. In agreement with this suggestion, one more recent study has demonstrated that the presence of both the MHR and adjacent C-terminal capsid sequences within the Gag-Pol proteins are required for the incorporation Pr160$^{gag\text{-}pol}$ into virus particles [Huang and Martin, 1997].

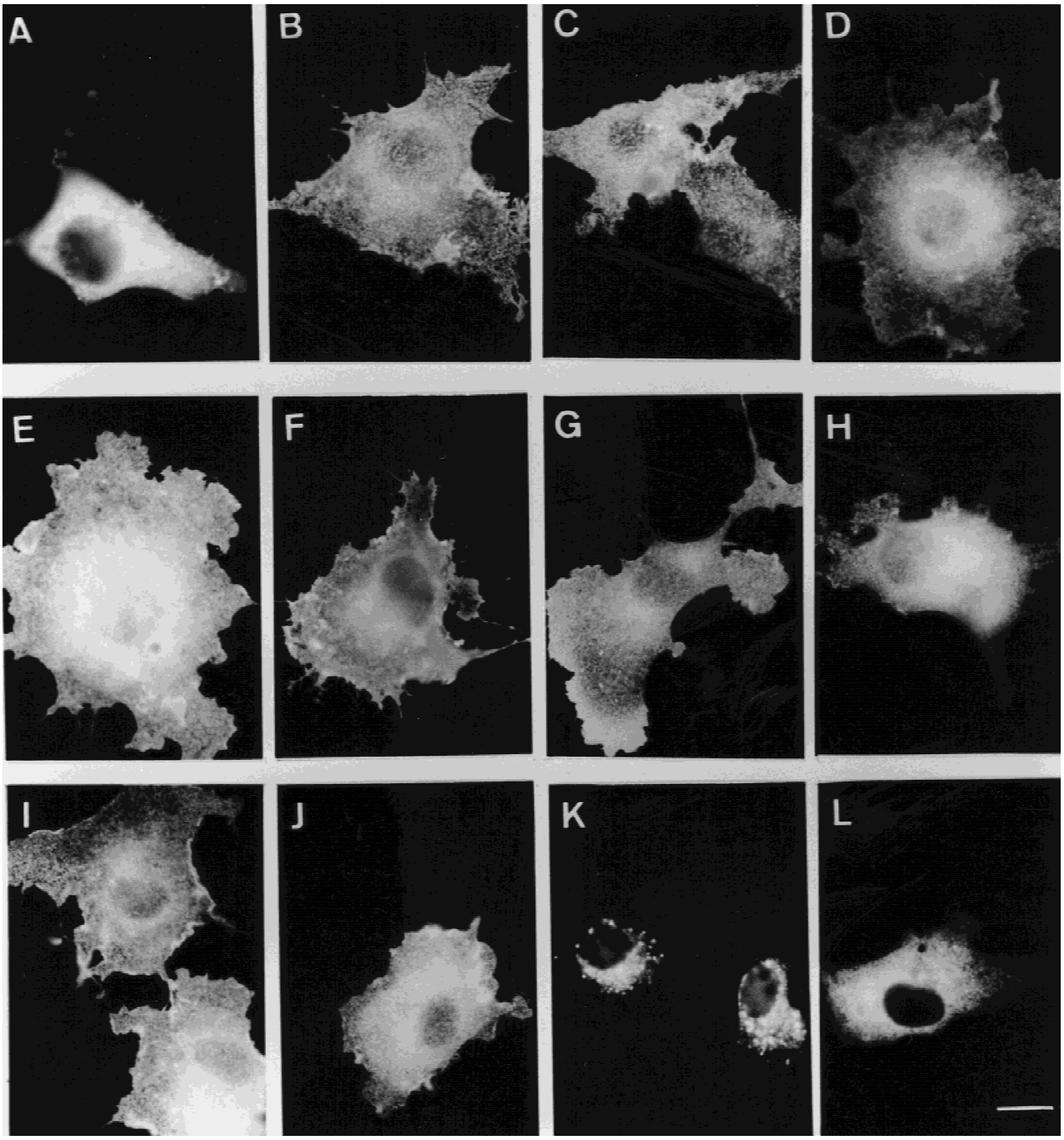

Fig. 4. Indirect immunofluorescence detection of HIV Gag-β-gal fusion proteins. COS7 cells grown on cover slips were transfected with HIV Gag-β-gal expression constructs HIVGBG **(A)**; GBG 1919 **(B)**; GBG 1877 **(C)**; GBG 1801 **(D)**; GBG 1752 **(E)**; GBG 1715 **(F)**; GBG 1600 **(G)**; GBG 1511 **(H)**; GBG 1447 **(I)**; GBG 1147 **(J)**; GBG 831 **(K)**; GBG Myr^- **(L)**. Two days after transfections, cells were fixed and permeabilized for immunofluorescence assays as described in the Materials and Methods section. B-gal fusion proteins were detected with a mouse anti-β-gal antibody at 1:3,000 dilution followed by a goat anti-mouse rhodamine-conjugated IgG antibody at 1:100 dilution. The white bar in (L) indicates 20 μm.

The data of β-gal release ratios (Table I) did not correlate with the results of subcellular fractionation patterns (Table II). Similarly, the levels of HIVgpt-induced Gag-β-gal release appeared not to correlate with the immunofluorescence localization staining patterns (Fig. 4). Fusion proteins with intact MHR but truncated N-terminal CA domains (GBG 1752 and 1715) were predominantly localized in the cytosol fractions. However, deletions of both the MHR and the adjacent C-terminal CA restored the fusion proteins' abilities (GBG 1684, 1600, 1511, 1447, 1419, 1147, and 831) to associate with intracellular membranes at a

level approximately halfway between the HIVGBG and GBG Myr$^-$. These results suggest that the MHR may have a deleterious effect on fusion protein intracellular membrane association when the adjacent C-terminal CA is deleted. Potentially relevant to intracellular transport were enhanced surface immunofluorescence staining patterns of fusion proteins (GBG 1919–1147) with intact MA but deleted NC domains. This staining pattern has also been observed in 293T cells expressing HIV Gag proteins with intact MA but deleted NC domains [Wang et al., 1998]. Whether this staining pattern is relevant to the mechanism of HIV assembly is unknown although it may suggest that the NC domain may increase Gag or Gag-β-gal fusion protein affinity for intracellular compartment.

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